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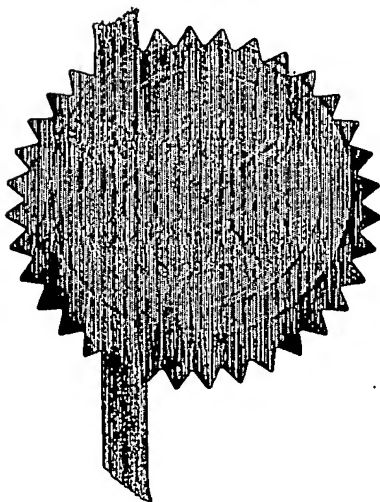
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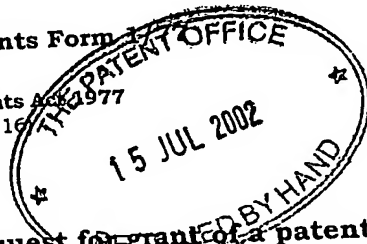
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1/77

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1. Your reference	H-32534P1		
2. Patent application number (The Patent Office will fill in this part)	0216414.3		15 JUL 2002 6JUL02 E733504-1 000524 P0177700 0.00-0216414.3
3. Full name, address and postcode of the or of each applicant (underline all surnames)	NOVARTIS AG LICHTSTRASSE 35 4056 BASEL SWITZERLAND 7125487 005 SWITZERLAND		
Patent ADP number (if you know it) If the applicant is a corporate body, give the country/state of its incorporation			
4. Title of invention	Organic compounds		
5. Name of your agent (if you have one)	B.A. YORKE & CO. CHARTERED PATENT AGENTS COOMB HOUSE, 7 ST. JOHN'S ROAD ISLEWORTH MIDDLESEX TW7 6NH		
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Patents ADP number (if you know it)		1800001	
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day/month/year)	
8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:	Yes		
a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is not named as an applicant, or c) any named applicant is a corporate body. (see note (d))			

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Description 21

Claim(s) 4

Abstract 1

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Sequence Listing pages 26-28

11.

I/We request the grant of a patent on the basis of this application

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Date

B.A. Yorke & Co.

B.A. Yorke & Co.

15 July 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

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020 8560 5847

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DUPLICATE

### Organic Compounds

#### Field of the Invention

The present invention relates to heat shock protein (hsp) genes and encoded proteins from *Corynebacteria*. In particular, it concerns the isolated DNA sequence and amino acid sequence of hsp70 from the genus *Arthrobacter*, and sequences homologous thereto. Further, the invention relates to uses of *Arthrobacter* hsp70 in the preparation of vaccines, especially vaccines for fish, as an adjuvant, and as a carrier for antigens.

#### Background of the Invention

A live, non-virulent strain of *Arthrobacter* (a member of the family of *Corynebacteria*) is marketed under the name "Renogen" in a vaccine intended to protect salmon and other farmed fish against bacterial kidney disease (BKD). The characteristics of this strain are disclosed in WO 98/33884. This vaccine is unique in that it is the first live culture to have been licensed for use in aquaculture.

Recently, use in the field has provided circumstantial evidence that the vaccine also confers a protective effect against salmonid rickettsial septicaemia (SRS). Furthermore, there are reports of non-specific immunostimulatory effects in vaccinated fish against opportunistic pathogens such as those responsible for motile aeromonad septicaemia.

We wished to identify *Arthrobacter* proteins which would exhibit a degree of immunogenicity comparable to that observed with the live bacterium. The intention was to develop a recombinant protein vaccine or DNA vaccine to protect against a wide variety of fish diseases.

A successful vaccine against intracellular pathogens will not only stimulate the humoral immune response via the Major Histocompatibility Complex (MHC) class II pathway, but (more importantly) will also induce destruction of infected cells through activation of the MHC class I pathway. The latter response is achieved through cytosolic degradation of foreign protein in infected cells, such that fragments of the foreign material are shuttled to the cell surface for presentation to CD8<sup>+</sup> cytotoxic T cells (CTL).

Heat shock proteins ("Hsps") are a family of molecular chaperone proteins produced by prokaryotic and eukaryotic cells, and which play essential roles in a multitude of intra- and intercellular processes, in particular in antigen processing and presentation of antigen fragments to the MHC I system at the cell surface. Fusion proteins of certain hsp proteins with other peptides have been successfully used *in vivo* to elicit a CTL response specific to those peptides.

Furthermore, hsps are now known to be targets of anti-pathogen immune responses against a multitude of bacterial, fungal, helminthic and protozoal diseases. Immunization of mammals with a variety of different pathogen hsps (principally of mycobacterial origin) induces strong immune responses and provides protection against diseases caused by these pathogens. The strength of immune responses to pathogenic hsps is probably due in part to the existence of multiple B cell and T cell epitopes on these proteins.

Based on this information, we hypothesized that hsp analogues in *Corynebacteria* such as *Arthrobacter*, and in particular those of the *Arthrobacter* strain used in the Renogen vaccine, might serve as suitable immunogens for vaccines against fish pathogens.

#### Summary of the Invention

In a first aspect, the invention provides an isolated nucleic acid molecule comprising the sequence of the *Arthrobacter* hsp70 gene, a fragment thereof, or a related sequence. The gene includes the ORF, 5'UTR and 3'UTR, and any component promoter, enhancer, regulatory, terminator and localization elements.

In a second aspect the invention provides an isolated amino acid sequence comprising the sequence of *Arthrobacter* hsp70 protein, a fragment thereof, or a related protein.

In another aspect, the invention provides a vaccine composition comprising a nucleic acid molecule encoding an *Arthrobacter* hsp70 protein or comprising a polypeptide molecule which is an *Arthrobacter* hsp70 protein. The vaccine composition can be used in the preparation of a medicament for human or veterinary use, most advantageously for use in aquaculture.

In a further aspect of the invention there is provided a nucleic acid molecule encoding a fusion protein of whole or part of the hsp70 protein of *Arthrobacter*, and a non-hsp70 antigen. Also provided is the fusion protein itself.

In yet another aspect of the invention there is provided an entity comprising a polypeptide comprising whole or part of the hsp70 protein of *Arthrobacter*, which is covalently attached to a non-hsp70 entity.

In a further aspect of the invention there is provided use of *Arthrobacter* hsp70 protein as a vaccine antigen, non-specific adjuvant, or as a carrier for heterologous molecules, with the aim of treating or preventing animal diseases.

In another aspect of the invention there is provided use of an *Arthrobacter* hsp70 promoter to drive expression of a heterologous gene.

#### Description of the Figures

Fig. 1 depicts SEQ ID NO:1, i.e. the DNA sequence (5' to 3') of the hsp70 gene isolated from *Arthrobacter* ATCC 55921, including 5' and 3' UTR sequences.

Fig. 2 shows SEQ ID NO:2, i.e. the amino acid sequence predicted to be encoded by the hsp70 gene sequence of Fig. 1.

#### Detailed description of the Invention

The novel sequences of the hsp70 gene of the invention and the encoded protein are provided in Figures 1 and 2, respectively. The invention encompasses nucleic acid sequences and amino acid sequences which are substantially homologous to the sequences provided in the Figures. "Substantially homologous" in this context means that a sequence, when compared to a reference sequence, has at least 60% homology, preferably at least 70% homology, more preferably at least 80% homology, more preferably at least 90% homology, and most preferably at least 95% homology to the reference sequence.

To determine the percent homology of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g. gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence and the intervening non-homologous sequence in the gap can be disregarded for comparison purposes). There is no requirement for the two sequences to be the same length. In general, the length of sequence across which the sequences are compared is the entire extent of the alignment. Optionally, the length of a reference sequence aligned for comparison purpose is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least, 70%, 80%, or 90% of the length of the reference sequence. It possible to restrict homology analysis to any particular portion of the reference sequence, e.g. in the case of hsp70 one might wish to restrict the reference sequence to the amino terminal half of the gene or protein, or more specifically to structural lobe II (Flaherty et al. (1990) Nature 346: 623-628 & Zhu et al. (1996) Science 272: 1606-1614).

When a position in the first (reference) sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the sequence, the molecules are homologous at that position (i.e. there is identity at that position). In the case of nucleic acid sequence comparison there is also homology at a certain position where the codon triplet including the nucleotide encodes the same amino acid in both molecules being compared, due to degeneracy of the genetic code.

The percent homology between two sequences is a function of the number of homologous positions shared by the sequences (i.e., % homology = no. of homologous positions/total no. of positions). Optionally, the comparison of sequences and determination of percent homology can be accomplished using a mathematical algorithm. Suitable algorithms are incorporated in to the NBLAST and XBLAST programs of Altschul et al. (1990) J. Mol. Biol. 215:430-10.

The definition of homologous sequences provided above embraces fragments of the reference nucleic acid sequence or amino acid sequence. For present purposes a "fragment" of a hsp70 protein is understood to mean any peptide molecule having at least 25, optionally at least 35, or at least 45 contiguous amino acids of the reference hsp70 amino acid sequence. A "fragment" of the hsp70 nucleic acid reference sequence is any part

of that sequence comprising at least 50, optionally at least 75, or at least 100 consecutive nucleotides.

Also comprised within the nucleic acid sequences of the invention are sequences which hybridize to the reference SEQ ID NO:1 under stringent conditions. "Stringent" hybridization conditions in the sense of the present invention are defined as those described by Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), 1.101-1.104, i.e. a positive hybridization signal is still observed after washing for 1 hour with 1x SSC buffer and 0.1% SDS at 55°C, preferably at 62°C and most preferably at 68°C, in particular for 1 hour in 0.2 x SSC buffer and 0.1% SDS at 55°C, preferably at 62°C and most preferably at 68°C.

The hsp70 nucleic acid sequences of the invention incorporate the Open Reading Frame (ORF) of the hsp70 gene, but also the 5' and 3' Untranslated Regions (UTR). The invention includes any component promoter, enhancer, regulatory, terminator and localization elements, and use of these elements in conjunction with heterologous genes.

The amino acid sequences of the invention also comprise derivatives of the sequence of Figure 2, or of homologues of that sequence. A "derivative" of an amino acid sequence is a sequence related to the reference sequence either on the amino acid sequence level (e.g. a homologous sequence wherein certain naturally-occurring amino acids are replaced with synthetic amino acid substitutes) or at the 3D level, i.e. molecules having approximately the same shape and conformation as the reference amino acid sequence. Thus, derivatives include mutants, mimetics, mimotopes, analogues, monomeric forms and functional equivalents. Amino acid sequence derivatives retain the ability to induce the production of antibodies that recognize and (cross)-react with the antigens from fish pathogens such as *P. salmonis* or *R. salmoninarum* and/or to induce an immune response in fish that protects against infection with these pathogens.

The *Arthrobacter* hsp70 sequence listing provided herewith (SEQ ID NO:1) is the sequence of the gene identified by genomic cloning to be present in *Arthrobacter*, and SEQ ID NO:2 is the amino acid sequence inferred therefrom. A culture of the source *Arthrobacter* strain was deposited under Accession No ATCC 55921 with the American Type Culture Collection on 20 December 1996.



The hsp proteins of species of the same genus are generally very highly conserved, so it is to be expected that the sequences of hsp70 genes and proteins native to other *Corynebacteria*, and especially other *Arthrobacter* species, will not diverge greatly from SEQ ID NO:1 and SEQ ID NO:2, respectively. Therefore the present invention extends also to these related hsp70 molecules. Knowledge of sequence of the hsp70 gene from one *Corynebacterial* species facilitates isolation of the same genes from related organisms. Procedures for isolation of these genes are well known in the art.

DNA encoding hsp70s may be obtained from a cDNA library prepared from cell matter expressing the hsp70 (hsps are ubiquitous and expressed in abundance). The hsp70 encoding gene may also be obtained from a genomic library, such as by following steps described in Example 1, or by oligonucleotide synthesis.

Native hsp70 proteins can be isolated from bacterial cell sources by an appropriate purification scheme using standard protein purification techniques. The identity of the protein can be confirmed, for instance, by Western blotting or immunoprecipitation using antibodies to *Arthrobacter* ATCC 55921 hsp70 antigen. N-terminal amino acid sequencing can be used to determine partial or complete amino acid sequences. This enables design of probes to facilitate isolation of the native hsp70 sequence from a cDNA or genomic library.

Libraries can be screened with probes (such as antibodies to the hsp70 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. For instance, the probes may be designed to be homologous to parts of the *Arthrobacter* gene sequence disclosed herein. Alternatively, the probes may have a high degree of homology with other bacterial hsp genes, such as the *Vibrio* hsp70 gene sequence. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding hsp70 is to use PCR methodology [Sambrook et al., *supra*; Dieffenbach et al., *PCR Primer: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1995)].

Sequences identified in such library screening methods can be compared and aligned to the hsp70 disclosed in SEQ ID NO:1 or other known hsp sequences deposited and available in public databases such as GenBank. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined through sequence alignment using computer software programs such as BLAST, BLAST-2, ALIGN, DNASTar, and INHERIT which employ various algorithms to measure homology.

The *Arthrobacter* hsp70 protein can be seen to bear a resemblance to homologues in other species, especially bacterial species. It is known that hsp genes are highly conserved between related species. However, it came as a surprise to discover that the *Arthrobacter* hsp70 exhibits near identity to the hsp70s from *Mycobacterium tuberculosis* and *Mycobacterium leprae* in the N-terminal region (the first 20 amino acids are in fact identical). We believe that the hsp70 from *Arthrobacter*, like *Mycobacterium* hsp70, is exceptionally immunogenic in its own right, and could account to some degree for the broad spectrum of disease protection conferred by the Renogen live *Arthrobacter* vaccine.

In related experiments, described in Example 2, an abundant *Arthrobacter* surface protein of about 67kDa that is apparently linked to cell wall peptidoglycan was analysed by N-terminal amino acid sequencing. The N-terminal amino acid sequence of 20 residues was found to be identical to that of *Mycobacterium* hsp70, and this 67kDa protein is believed to correspond to the hsp70 protein of the present invention.

The realization that *Arthrobacter* and *Mycobacterium* share highly homologous hsp70 genes opens up many unforeseen applications for the *Arthrobacter* homologue. Isolated *Mycobacterium* hsp70 protein has been demonstrated to perform as an effective vaccine adjuvant. *Arthrobacter* hsp70 can also be used as an adjuvant in pure or isolated form in conjunction with an antigen. An "adjuvant" as defined herein is a substance that nonspecifically augments the specific immune response to an antigen when mixed with the antigen prior to administration, or when administered separately into the same site. In one aspect of the invention isolated or purified *Arthrobacter* hsp70 protein is used as a general (non-specific) adjuvant for an animal vaccine. In a related application, the hsp70 gene or a portion thereof is provided on a DNA vector in order to adjuvant a nucleic acid vaccine. Within the scope of the invention there are provided vaccine compositions comprising the

amino acid or nucleic acid sequences of the invention in conjunction with at least one other antigen or antigen-encoding nucleic acid sequence, and one or more pharmaceutically acceptable excipients. The other antigen may be a recombinant or isolated single antigen, or it may be a mixture of antigen molecules from a pathogen.

The use of *Arthrobacter* hsp70 as an adjuvant allows doctors and veterinarians to move away from use of the traditional attenuated live *Mycobacterial* adjuvants, which present a risk to the health of animals due to the danger of reversion to the virulent bacterial strain. There is an additional benefit for aquaculture in that injection of *Arthrobacter* hsp70 nucleic acid or isolated protein into fish does not result in disfiguring swellings or nodules at the injection site, which are common with conventional adjuvants and which lower the commercial value of the fish.

*Arthrobacter* hsp70 protein is not only effective in adjuvanting vaccines comprising other antigens, but it also has immunogenic activity in its own right. *Arthrobacter* hsp70 can provide the active principle for a vaccine to prevent or treat a variety of human and veterinary diseases, including diseases caused by fish pathogens, in particular, but not limited to, SRS and BKD. In a further aspect of the invention a vaccine composition comprises isolated or purified hsp70 protein as the sole antigenic or immunogenic component, together with one or more pharmaceutically acceptable excipients. Also provided is a nucleic acid vaccine composition comprising an expression vector comprising a nucleic acid sequence of the present invention encoding an antigen being the sole antigenic or immunogenic component of the vaccine composition, together with one or more pharmaceutically acceptable excipients.

The highly immunogenic potential of *Arthrobacter* hsp70 deduced from homology to the *Mycobacterium* and other hsp70s also suggests the possibility of preparing gene or peptide covalent conjugates (e.g. chimeras or fusions) of a hsp70 protein with a heterologous (non-hsp70) molecule, usually, but not limited to, a non-hsp protein (e.g. a hapten). In this manner, the *Arthrobacter* hsp70 protein acts as an adjuvant-free carrier to stimulate the humoral and cellular immune responses to the accompanying heterologous molecule. The carrier serves to non-specifically stimulate T helper cell activity and to help direct the molecule to antigen presenting cells for processing and presentation of the antigen fragments at the cell surface in association with molecules of the MHC I complex. This

approach to vaccine development is particularly advantageous when the antigenic peptide concerned is not very large and poorly immunogenic, yet would be a suitable target for a vaccine. The heterologous molecule carried by the *Arthrobacter* hsp70 is advantageously a protein hapten or a non-protein molecule such as a carbohydrate moiety. As used herein the term "carrier" refers to a molecule containing T cell epitopes which, when covalently linked to a second molecule, helps to elicit and enhance immune responses against the second molecule (which may be a protein, peptide or an oligosaccharide).

As used herein, a hsp "fusion protein" comprises a hsp polypeptide operatively linked to a different polypeptide (a "heterologous polypeptide"). A "heterologous peptide" or a "non-hsp70 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the hsp70 protein. Within a hsp70 fusion protein the hsp70 polypeptide can correspond to all or a portion of a hsp70 protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the hsp70 polypeptide and the non-hsp70 polypeptide are fused in-frame to each other. The non-hsp70 polypeptide can be fused to the N-terminus or C-terminus of the hsp70 polypeptide, or can be embedded within the hsp70 polypeptide.

It is possible to link a hsp70 polypeptide and a heterologous molecule by means of a covalent linkage other than by creating a fusion protein. For instance, chemical spacer groups may be inserted between polypeptides, e.g. to create a molecule of general formula hsp70 -X-heterologous polypeptide, where X is a spacer group, such as a short sequence of one or more amino acids. Alternatively, the hsp70 polypeptide may be covalently linked other than through amide linkages in a linear chain of amino acids, for instance by chemical conjugation or by chemical or radiation-induced crosslinking to the non-hsp molecule. Glutaraldehyde is an example of a suitable chemical cross-linker.

In a preferred embodiment of the invention, the hsp70 polypeptide is conjugated to a hapten. A hapten is a substance of low molecular mass (e.g. a peptide or oligosaccharide) that can bind antibodies, but which will induce an immune response only if covalently attached to a large carrier molecule.

The isolated hsp70 gene from *Arthrobacter* can be exploited in the conventional manner, by cloning the gene into an expression vector for generation of large quantities of purified or

isolated recombinant hsp70 protein (or hsp70 fusion protein). A purified hsp70 antigen can also be obtained by non-recombinant techniques. The protein is abundant and can be extracted from cells by conventional purification methods. Alternatively, the hsp70 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

A vaccine comprising this purified or isolated recombinant or non-recombinant protein can be termed an antigen-based vaccine.

An "isolated" or "purified" protein is defined as being substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the hsp70 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of hsp70 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of hsp70 protein having less than about 30% (by dry weight) of non-hsp70 protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-hsp70 protein, still more preferably less than about 10% of non-hsp70 protein, and most preferably less than about 5% non-hsp70 protein. When the hsp70 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

Alternatively, the hsp70 gene can be incorporated into a Nucleic Acid Vaccine (NAV), whereby the NAV is taken up by host cells of a living animal, and expression of the hsp70 gene takes place within the cytosol. Because short peptides of intracellular hsp70 antigens are transported to the cell surface where they can make contact with the MHC I system, NAV-originating hsp70 antigens are ideally positioned for inducing a cellular immune response.

A hsp70 gene inserted into a DNA vector can be inoculated directly into a fish (e.g. orally, intramuscularly or intraperitoneally) for expression in vivo within fish cells. DNA vaccination can also be carried out in other animal species. Thus, in one aspect of the invention there is provided a nucleic acid vaccine comprising a pharmaceutically acceptable carrier and a DNA plasmid in which a nucleic acid sequence encoding *Arthrobacter* hsp70 is operably linked to

a transcriptional regulatory sequence. Transcriptional regulatory sequences include promoters, polyadenylation sequences and other nucleotide sequences such as the immune-stimulating oligonucleotides having unmethylated CpG dinucleotides, or nucleotide sequences that code for other antigenic proteins or adjuvanting cytokines. For optimal in vivo expression it may be preferred to select transcriptional regulatory sequences endogenous to the fish to be vaccinated. For instance, endogenous cytokine or actin gene promoters may be considered. The DNA can be present in naked form or it can be administered together with an agent facilitating cellular uptake (e.g. liposomes or cationic lipids). The technology of DNA vaccination of fish is explained in more detail in US 5,780,448, which is incorporated herein by reference.

Preferably, a hsp70 chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). A hsp70-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the hsp protein.

Another aspect of the invention pertains to vectors, preferably expression vectors, comprising a nucleic acid sequencing encoding hsp70 (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of directing the expression of genes to which they are

operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, operatively linked to the nucleic acid sequence to be expressed. Recombinant expression vectors of the invention may be used for expression within the intended recipient of the hsp70 antigen (as a DNA vaccine) or for expression within a host organism other than the final recipient (for production of recombinant antigen vaccines).

Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g. hsp70 proteins, mutant forms of hsp70, fusion proteins of hsp70 with a heterologous peptide, etc.).

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. A host cell can be any prokaryotic or eukaryotic cell.

For example, hsp proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Other suitable host cells are known to those skilled in the art (e.g. Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990)). The recombinant expression vector may be designed to be expressed in a host fish cell (follow DNA vaccination). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D. B. and Johnson, K. S. (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

The present invention also relates to a method of generating monoclonal or polyclonal antibodies to a molecule using a conjugate of a hsp70 protein joined to the molecule. In this embodiment, an effective amount of the conjugate (i.e., an amount which results in an immune response in the host) is introduced into an animal host which results in production of antibodies to the substance in the host. The antibodies are removed from the host and purified using known techniques (e.g. chromatography), thereby resulting in production of polyclonal antibodies. Alternatively, the antibodies produced using the method of the present invention can be used to generate hybridoma cells which produce monoclonal antibodies using known techniques.

In one embodiment of the invention the promoter sequence of the *Arthrobacter* hsp70 gene is used to drive expression of a heterologous gene, i.e. a gene other than the gene encoding *Arthrobacter* hsp70. The promoter can be inserted upstream of a heterologous gene in the



chromosomal DNA of an organism, or into an extrachromosomal plasmid or other expression vector. For instance, in the event that it is desired to over-express an endogenous *Arthrobacter* gene, or to insert a foreign gene into an endogenous plasmid of *Arthrobacter*, an upstream hsp70 promoter can drive expression of that heterologous gene in response to a stimulus such as heat shock.

The vaccines manufactured in accordance with the methodology of the invention are suited for administering to any species of animal having a humoral and/or cellular immune system comparable to that of mammals. Humans are included within the meaning of "animal" and "mammal" in the present context. *Arthrobacter* hsp70 can be employed to adjuvant any vaccine for mammals, birds, reptiles or fish. Similarly, *Arthrobacter* hsp70 can be used in vaccines as carriers for covalently-attached antigens, optionally to the exclusion of any conventional adjuvant (so-called "non-adjuvant" vaccines). *Arthrobacter* hsp70 is also capable of being used as an immunogen (optionally as the sole immunogen) in a vaccine for raising an immune response against specific diseases, notably SRS, BKD and other infectious diseases in fish.

The term "vaccine" is used in the broad sense, and includes not only compositions to be used for immunization against pathogens, but also anti-tumor vaccines, vaccines based on autogenous antigens (e.g. for chemical castration), and so on. Within the sphere of veterinary vaccination, the major species of land animals to be considered for immunization include cattle, horses, sheep, swine and poultry birds. For aquaculture, the vaccines of the invention can be employed in treatment of teleosts such as salmon, trout, carp, sea bream, sea bass, yellowtail, catfish, halibut, haddock, or optionally for treatment of other aquatic species such as crustaceans and mollusks. There are no limits to the candidate antigens suitable for combining with *Arthrobacter* hsp70 sequences in a vaccine. Pathogenic antigens can be derived from bacteria, viruses, protozoa, nematodes and fungi. One particular focus is on antigens, particularly surface antigens, of fish pathogenic organisms.

Therapeutic or preventative vaccination can be employed against bacterial, protozoan, viral or fungal diseases in teleosts. Disease-causing organisms in fish against which vaccination is desired to be effective include Infectious Salmon Anaemia Virus (ISAV), Infectious Pancreatic Necrosis Virus (IPNV), Infectious Hematopoietic Necrosis Virus (IHNV), Iridovirus, Nervous Necrosis Virus (NNV), Salmon Pancreas Disease Virus (SPDV), Spring

Viremia of Carp Virus (SVCV), Viral Hemorrhagic Septicemia Virus (VHSV), *Renibacterium salmoninarum* (causative agent of Bacterial Kidney Disease), *Piscirickettsia salmonis* (causative agent of Salmonid Rickettsial Septicemia), *Vibrio* spp, *Aeromonas* spp, *Yersinia ruckerii*, *Pseudomonas* spp., *Photobacterium damsela*, etc.

-Hsp70 sequences can also be used in conjunction with antigens from other animal pathogens and parasites, including: Bovine Viral Diarrhea Virus (BVDV), BHV, Foot and mouth disease virus, Bovine Respiratory Syncytial Virus (BRSV), Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), *Mycobacteria*, *Leishmania*, *Eimeria*, *Clostridia*, *Pasteurella*, *Mycoplasma*, *Leptospira*, *Salmonella*, *Brucella*, *Neospora*, *Cryptosporidium*, *Fusobacterium*, *E. coli*, *Rotavirus*, *Coronavirus*, *Mannheimia haemolytica*, *Haemophilus somnus*, *Actinobacillus pleuropneumoniae*, *Trypanosoma*, *Anaplasma*, etc.

The vaccine antigens provided in conjunction with hsp70 gene or protein may be chemically conjugated to the hsp70 (in a chimera or fusion protein) or they may be provided as separate molecules together in a single vaccine composition. As another option, hsp70 gene or protein may be provided with an antigen or antigen-encoding nucleic acid sequence in a kit for separate, sequential or simultaneous administration.

It is possible to immunize a subject with the neutral or the salt forms of the present fusion proteins or isolated hsp70 protein, either administered alone or in admixture with a pharmaceutically acceptable vehicle or excipient. Typically, vaccines are prepared as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to administration may also be prepared. The preparation may be emulsified or the active ingredient encapsulated in liposome vehicles. The pharmaceutical compositions of the invention may be administered in a form for immediate release or by extended release.

Pharmaceutically acceptable vehicles are, for example, water, saline, dextrose, glycerol, auxiliary substances such as wetting or emulsifying agents, bulking agents, binders, disintegrants, diluents, lubricants, pH buffering agents, or conventional adjuvants such as muramyl dipeptides, avridine, aluminium hydroxide, oils, saponins, block co-polymers and other substances known in the art.

To immunize a subject, a hsp70 antigen or hsp70 gene vector can be administered parenterally, usually by intramuscular injection in an appropriate vehicle, but optionally by the subcutaneous route, by intravenous injection or by intradermal or intranasal delivery. In the case of immunization of fish, the typical routes of administration are by injection into the peritoneal cavity, orally in feed, or by immersion.

The effective dosage may vary depending on the size and species of the subject, and according to the mode of administration. The optimal dosage can be determined through trial and error by a doctor or veterinarian. Typically, a single dose of hsp70 antigen will be in the range of from about 0.01 to 1000 $\mu$ g per kg body weight, preferably 0.5 to 500  $\mu$ g per kg, more preferably 0.1 to 100  $\mu$ g per kg. For DNA vaccines, a minimum dosage of 10pg up to dosages of 1000 $\mu$ g of plasmid per animal should be sufficient for suitable expression of the antigen in vivo.

The novel antigens disclosed as part of the present invention are also useful in screening for antibodies to pathogenic proteins. The invention additionally includes diagnostic uses of these antigens, for instance in the preparation of a diagnostic kit, useful for testing animals for the presence of disease-causing organisms.

It is also contemplated that antibodies raised against the purified antigens of the invention can have both diagnostic and therapeutic applications in disease management. Both polyclonal antibodies and monoclonal antibodies may be useful in this respect. Procedures for immunizing animals, eg. mice, with proteins and selection of hybridomas producing immunogen-specific monoclonal antibodies are well known in the art (see for example Kohler and Milstein (1975) *Nature* 256: 495-497). Sandwich assays and ELISA may be mentioned as specific examples of diagnostic assays.

#### Examples

Example 1: Isolation and sequencing of hsp70 gene from the genome of *Arthrobacter* ATCC 55921

A 5ml culture of *Arthrobacter* was grown overnight shaking at 30°C in LB containing kanamycin (30µg/ml). DNA extraction was then carried out using the Puregene DNA isolation kit (Gentra) or Instagene<sup>TM</sup> Resin according to the manufacturer's instructions.

#### Degenerate PCR

Areas of greatest similarity between several mycobacterial and streptomyces hsp70 (dnaK) sequences at the nucleotide level were used to design degenerate primers for PCR and sequencing. The selected primers were dnaK-1Fdeg (5'-gtcggnatcgacctvggnac-3') and dnaK-4Rdeg (5'-gctggtsggctcgttgac-3'). These primers were used for amplification of *Arthrobacter* DNA in a PCR reaction with a 50°C annealing temperature. The quality of the amplified DNA was assessed by gel electrophoresis. A 10µl aliquot was electrophoresed on a 0.8% agarose gel in 1X Tris-borate electrophoresis buffer (TBE) at 100V for about 1 hour. The 650bp product was then excised from the gel and purified using the Qiaquick purification kit (Qiagen). The PCR product was cleaned using Qiagen PCR clean up kit according to the manufacturer's instructions) and sequenced according to the manufacturer's instructions using BigDye primer chemistry (Applied Biosystems) and each of the primers used for the PCR. Briefly, the extension reaction mixtures were prepared using the ABI PRISM (8r) BigDye Terminator Cycle Sequencing Ready Reaction mix, ~ 600ng of DNA template, 3.2 pmol of the appropriate primer and ddH<sub>2</sub>O to 20 µl. Conditions for cycle sequencing were as follows: the thermal cycler was set to 25 cycles consisting of 96°C for 10s, 50°C for 5 s, and 60°C for 4 min. The sequence showed this fragment to contain the first approx. 400bp of the dnaK gene.

In order to obtain additional hsp70/dnaK gene sequence, degenerate primers were used to amplify a downstream portion of the *Arthrobacter* hsp70 gene. These were selected from Galley et al. (1992) Biochemica et Biophysica Acta 1130: 203-208. [Forward primer hsp70 universal-F1 (5' CAR GCN CAN AAR GAY GCN GG 3'), Reverse primer hsp70 universal-R1: (5' GCN CAN GCY TCR TCN GGR TT 3')]. The primers were designed to anneal to two highly conserved regions within the hsp70 gene to generate a 650bp product.

The PCR reaction consisted of (in 50 µl total volume): 5µl 10X PCR buffer, 5µl 25mM MgCl<sub>2</sub>, 2.5µl 10mM each dNTPs, 2µl Hsp70 universal-F1 (200µM), 2µl Hsp70 universal-R1 (200µM), 0.5 µl Amplitaq DNA polymerase (5 U/µl), 10µl of Instagene extracted DNA, 23µl

ddH<sub>2</sub>O. The PCR reaction was cycled as follows: 2 min at 94°C, then 40 PCR cycles consisting of: 1.0 min at 94°C, 0.5 min at either 50°C or 58°C, and 1.0 min at 72°C. PCR cycling was completed with a 3.0 min elongation step at 72°C. The PCR product was electrophoresed, cleaned and sequenced as described above. The sequence of this fragment was found to overlap and extend 3' of the ~400bp sequence identified in the first sequencing attempt.

### Genome Walking

Genome walking was used to extend the sequence 5' and 3' of the already identified sequences. Primers were designed from the sequences described in the Genome Walker manual (Clontech). Initially, four genomic libraries were made (DraI, EcoRV, PvuII, StuI), and subsequently three additional libraries were made (AluI, ScaI, and SnaBI). All libraries and the subsequent PCR reactions were as described in the Genome Walker manual.

5' Genome walking produced a ~500bp fragment which overlapped with the sequence previously obtained. The 5' extension contained the start codon and the 5' UTR of the hsp70 gene. 3' walking failed to extend the gene.

### RACE

3' RACE was used instead to obtain additional 3' sequence. RNA was isolated from an overnight culture of *Arthrobacter* (cultured as above) using the Purescript (Gentra) RNA isolation kit according to the manufacturer's instructions. 3' RACE was performed using the Boehringer Mannheim 5'3' RACE kit according to manufacturer's instructions. Primers for RACE were designed from the 3' end of the known sequence. The PCR reaction was performed as described except that it contained 10% DMSO and a 55°C annealing temperature. A band of ~1.0kb was amplified and cloned into a pCR4 vector using Invitrogen's TOPO sequencing kit according to the accompanying protocol. Colonies were screened using vector primers and select clones were grown overnight, then DNA extractions were carried out using Invitrogen's SNAP miniprep kit and sequenced using vector primers as described above.

Contigs were assembled using Genecode's Sequencher software. A Genbank search using the contigged sequences confirmed it was hsp70

The remainder of the hsp70 gene and 3'UTR was obtained by genome walking as described above. Again, contigs were identified using Genecode's Sequencher software. A Genbank search using the contigged sequences confirmed it included the hsp70 gene including 3' UTR and 5' UTR.

#### Example 2: Characterization of *Arthrobacter* cell wall proteins

##### Electrophoresis and Western Blotting

*Arthrobacter* ATCC 55921 cell suspensions were prepared by removing subcultured bacteria directly from tryptic soy agar (TSA) plates following incubation for 48 hours at 23°C. Bacteria were resuspended in 10ml of "TET" buffer consisting of 100mM Tris-HCl buffer of pH 7.2, 1mM EDTA and 0.1% Triton-X 100 (BioRad Laboratories, Hercules, CA), to an optical density of approximately 50 OD660 units (where 1 OD600 has been estimated to be  $1 \times 10^9$  cells), and centrifuged at 6500g. The supernatant was discarded and the cells were suspended in a further 10ml of TET and the centrifugation step was repeated. The cell pellet was resuspended in 4ml of TET and separated as 1 ml aliquots into 1.5ml microtubes. One of the aliquots was further mixed with 200µl of 5mg ml<sup>-1</sup> chicken egg white lysozyme and incubated with shaking overnight (18h) at 37°C. The cell suspension was centrifuged at maximum speed for 5 min in a bench top microcentrifuge. 150µl of cell free supernatant was mixed with 50µl of NuPage 4X LDS samples buffer (Invitrogen Carlsbad, CA) and incubated at 70°C for 10 min with shaking. 10µl aliquots of the resulting supernatant were analysed on 8% Mini Protean II gels (Biorad) and Western blotted to PVDF (Millipore, Bedford MA) with a semi-dry transfer unit (BioRad) for 30 min at 20V. Immediately after blotting, membranes were washed in 0.1% Ponceau S (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 min and destained with several washes of distilled water until blotted proteins were visible. Molecular weight standards and certain proteins were marked with pencil for reference points following immunostaining before removal of pink coloration by repeated washes in distilled water and air drying the membrane in preparation for further staining.

For Coomassie blue identification of protein bands for amino acid sequencing the decolorized membranes were incubated in a solution containing 50% methanol, 7% acetic

acid, and 0.1% of R-250 for 10 min followed by destaining of background using the aforementioned solution without Coomassie blue. The blot was subsequently rinsed in distilled deionised water for 10 minutes and air dried for N-terminal amino acid sequencing by Edman degradation using an automated protein sequencer (Applied Biosystems).

Development of immunoreactive antigens was achieved by incubation of the dried blot with a 1 in 200 dilution of antibody (polyclonal rabbit anti-*Arthrobacter*) in 1% casein tris-buffered saline pH 7.4 (cTBS) (BioRad) for 60 min, 2 washes of 10 sec with TBS followed by incubation with 1:2000 dilution of goat anti-rabbit immunoglobulin-alkaline phosphatase (Pierce, Rockford IL). Colour development was achieved with 1-Step NBT/BCIP (Pierce) for approximately 3 min and terminated by washing in distilled water. All incubations were carried out in a final volume of 10ml on a red rocker platform (Hoefer, San Francisco, CA). Documentation was achieved with an Imagemaster and associated software (Amersham Pharmacia Biotech, Uppsala Sweden). Estimation of molecular weights was achieved through comparison of migration of antigens with Precision broad range prestained standards (BioRad). Estimation of molecular weight was achieved by calculation of relative mobility using First Order Lagrange.

#### Antiserum Production

Polyclonal antiserum to *Arthrobacter* antigens was produced by i.m. injection of a New Zealand rabbit with 0.5ml of a suspension consisting of 3 parts  $4 \times 10^8$  cells previously washed in sterile Dulbecco's saline and 1 part Freund's incomplete adjuvant. After 4 weeks the same volume of material was injected into the rabbit. 5ml of blood was harvested from the peripheral ear vein at 6 weeks and serum collected by allowing blood to clot followed by centrifugation.

#### Results

SDS-PAGE analysis of the lysozyme treated suspensions of *Arthrobacter* revealed the presence of a range of protein bands between 150 and 10kDa. Preeminent among these were major proteins of approximately 67, 63 and 59kDa. The 67kDa protein is apparently cross-linked to the peptidoglycan of the *Arthrobacter* cell wall, because it is only released following treatment with lysozyme.

When the SDS-PAGE protein profiles were Western blotted and stained with an antiserum to whole cells of *Arthrobacter major* immunoreactive bands were identified at 122 and 67 kDa. The N-terminus of the 67kDa protein was sequenced and found to be SRAVG IDLGT JNSVV SVLE. A homology search using the BLAST protein-protein algorithm identified that the sequence had 100% homology to the hsp70 protein of *Mycobacterium tuberculosis*. This 67kDa protein is believed to be the same as the hsp70 protein identified by genomic sequencing and disclosed herein.



Claims

1. An isolated nucleic acid molecule encoding *Arthrobacter* hsp70, or a fragment thereof.
2. An isolated hsp70 nucleic acid molecule according to claim 1 which is from the *Arthrobacter* strain deposited under accession number ATCC 55921.
3. An isolated nucleic acid molecule, comprising the nucleic acid sequence of SEQ ID NO:1, or a fragment thereof, or a sequence substantially homologous thereto, or a sequence which (under stringent conditions) hybridizes with the sequence of SEQ ID NO:1.
4. An isolated nucleic acid molecule according to claim 3, comprising a sequence having at least 80% homology to SEQ ID NO: 1.
5. A chimeric nucleic acid molecule comprising the isolated nucleic acid molecule of any of claims 1 to 4 fused in-frame to a heterologous coding sequence.
6. A chimeric nucleic acid molecule according to claim 5, wherein said heterologous coding sequence encodes an antigen from an animal pathogen.
7. A DNA expression vector comprising the nucleic acid molecule of any of claims 1 to 6.
8. An isolated *Arthrobacter* hsp70 amino acid sequence, or a fragment thereof.
9. An isolated hsp70 amino acid sequence according to claim 8 which is from the *Arthrobacter* strain deposited under accession number ATCC 55921.
10. An isolated amino acid sequence comprising the amino acid sequence of SEQ ID NO:2, or a fragment thereof, or a sequence substantially homologous thereto, or a derivative thereof.

11. An isolated amino acid sequence according to claim 10 comprising a sequence having at least 80% homology to SEQ ID NO:2.
12. An amino acid sequence according to claim 10 or claim 11 which is covalently linked to a heterologous molecule to form a conjugate molecule.
13. An amino acid sequence according to claim 11 wherein said conjugate molecule is a fusion protein.
14. A fusion protein according to claim 13 wherein said heterologous molecule is selected from bacterial, viral, fungal, protozoan, nematode and tumour antigens.
15. An isolated amino acid sequence encoded by the nucleic acid molecule of any of claims 1 to 6.
16. An isolated nucleic acid molecule encoding the isolated amino acid sequence of any of claims 8 to 13.
17. A vaccine composition comprising the nucleic acid molecule of any of claims 1 to 6, and a pharmaceutically acceptable carrier.
18. A vaccine composition comprising the amino acid sequence of any of claims 8 to 13, and a pharmaceutically acceptable carrier.
19. A vaccine composition according to claim 17 or claim 18 and further comprising at least one heterologous antigen or a nucleic acid sequence encoding a heterologous antigen.
20. A kit comprising a vaccine composition according to claim 17 or claim 18 and a heterologous antigen or a nucleic acid sequence encoding a heterologous antigen, for separate, sequential or simultaneous administration.
21. Use of an amino acid sequence according to any of claims 8 to 11 as a non-specific vaccine adjuvant.

22. Use of a nucleic acid sequence according to any of claims 1 to 4 as a non-specific adjuvant for a nucleic acid vaccine.
23. A method of adjuvanting a vaccine comprising mixing a vaccine antigen with an amino acid sequence according to any of claims 8 to 11.
24. An antibody raised against the amino acid sequence of any of claims 8 to 13.
25. Use of a nucleic acid sequence according to any of claims 1 to 6, or an amino acid sequence according to any of claims 8 to 13, as a medicament.
26. Use of a nucleic acid sequence according to any of claims 1 to 6, or an amino acid sequence according to any of claims 8 to 13, in the preparation of a medicament for the immunization of an animal against infectious disease.
27. Use according to claim 26 wherein said animal is a teleost fish.
28. Use according to claim 27 wherein said medicament is a vaccine for prevention or treatment of Bacterial Kidney Disease (BKD) or Salmonid Rickettsial Septicaemia (SRS).
29. An isolated heat shock protein of approximately 67kDa measured by SDS-PAGE which is localized to the cell wall of *Arthrobacter* cells and has the N-terminal amino acid sequence: (M)SRVVG IDLGT TNSVV SVLE.
30. A method of inducing or enhancing an immune response to an immunogen or a hapten in an animal, the method comprising administering to said animal a pharmaceutical composition comprising a conjugate molecule according to claim 12 or claim 13, wherein the heterologous molecule of said conjugate molecule comprises said immunogen or hapten.
31. A method of therapeutic or prophylactic treatment of infectious disease in a fish, comprising administering to said fish a treatment composition comprising a nucleic acid molecule according to any of claims 1 to 6, or an amino acid sequence according to any of claims 8 to 13.

H-32534

- 25 -

32. A nucleic acid sequence having at least 70% homology with the promoter sequence of SEQ ID NO: 1.

33. A DNA expression vector comprising the nucleic acid sequence of claim 32, linked to a heterologous gene.

34. Use of the nucleic acid sequence of claim 32 to drive expression of a heterologous gene *in vivo*.

Abstract

The hsp70 gene from an *Arthrobacter* species has been isolated and sequenced. The encoded protein is believed to be highly immunogenic, especially in fish, and also has utility as a non-specific adjuvant, and as an adjuvanting carrier for heterologous antigens.

Fig.1 SEQ ID NO:1. *Arthrobacter* hsp70 DNA sequence (5' to 3'), 2464 nt including 5' and 3' UTR. The predicted ORF (nt 291-2156) is underlined

CTGCGAATGTCCACGTGGTGCCTGACGTGATGCGCTTGAAGGGCATCGCACCGCGCTGAA  
CCGGGTTTCGACCCGGTCCCACTGAGTTCGCCAACTGAGTGGGACAAGCCCGTTCTGTCCC  
AGTCACGCGGTCTGACTCAGTGGGACCACGCCGACGCGATCGATGGTCGCCGCACAGC  
TTTTTCCAAAGTTGAGCACAGGTGGCTCAACTTAGACTTGACATTGGTCGGCTCAAGCGT  
AAAGTTGATATCAGAACACTCAACTTGTAAGAAATCCCGAAAGGAAAAACATGTCAAG  
TGCAGTAGGCATCGACCTCGGAACCACTCGGTGGTTTCCGTCCTCGAAGGCGGCGA  
GCCCGTCGTATCGCGAACGCCGAAGGCGGCGCCACCAACCCCTCAGTCGTTCGGTTCTC  
CAAGAGCGGTGAAGTCCTGGTCGGCGAAATCGCCAAGCGCCAGGCCGTCAACAACATCG  
ATCGCACCATCGCCTCGGTCAAGCGCCACATGGGCACCGACTGGACCGTCGGCATCGACG  
ACAAGAAGTACACCGCGCAGGAAATCTCCGCCCGCACCTGATGAAGCTCAAGAACGAC  
GCCGAGTCCTACTTGGGCGAAAAGGTACCGACGCGGTGATCACGGTTCCTGCCTACTTC  
AACGACGCCGAGCGCCAGGCCACCAAAGAAGCCGGTGAGATCGCCGGCCTGAACGTGCT  
GCGCATCGTCAACGAGCCCACTGCGGCGGCGCTGGCCTATGGCTTGGACAAAGGCAAAG  
AAGACGAACTCATCTGGTCTTCGACCTCGGTGGCGGCACCTTCGACGTCTCGCTGCTGG  
AAGTCGGCAAAGACGACGACGGCTTCTCCACGATCCAGGTCCGCGCCACCTCCGGCGAC  
AACCGCCTGGGCGGCGACGACTGGGATCAGCGGATCGTCGACTACTTGCTGAACCAGCTC  
AAGGTCAAGGGCATCGACCTCTCCAAGGACAAGATCGCGCTGCAGCGTCTGCGCGAAGC  
TTCCGAGCAGGCCAAGAAGGAACTCTCCTCGGCCACCAGCACCAACATCTCGCTGCAGTA  
CCTCTCGGTACCCCTGACGGTCCGGTGCACTTGGACGAGCAGCTGACCCGGGCGAAGTT  
CCAGGAACTGACCGCTGATCTGCTCGAGCGCACCAAGAAGCCGTTCCAGGACGTGATCGC  
CGAGGCCGGGATCAAGGTTTCCGACATCGACCACATCGTGCTGGTCGGCGGTTCCACCCG  
GATGCCCGCAGTGACCGAATTGGTCAAGCAGCTGGCCGGTGGCAAGGAGCCGAACAAGG  
GCGTCAACCCGGACGAGGTGGTCGCCGTGCGCGCCGCGCTGCAAGCCGGCGTGCTCAAG  
GGCGAACGCAAAGACGTGCTGCTCATCGACGTACCCCGCTTTCCCTCGGCATCGAAACC  
AAGGGCGGCGTGATGACCAAGCTGATCGAGCGGAACACCGCGATTCCGACCAAGCGGTC  
CGAGACCTTCACCACGGCGGACGACACACCGCTTCGGTGGCCATCCAGGTGTTCCAAGG  
CGAGCGCGAGTTCACCCGGGACAACAAGCCGTTGGGCACCTTCGAACTGACCGGCATCG  
CACCGGCTCCGCGCGGCGTGCCGCAAGGTGGAAGTCACCTTCGACATCGACGCCAACGGCA  
TCCTGCACGTGTGCGGCCAAAGACAAGGGCACCGGCAAGGAGCAGTCGATGACCATCACC

GGCGGTTCCCTCGCTGTCCAAGGAAGACATCGAGCGCATGGTCGCCGACGCCGAGGCACA  
CGCTGCAGAGGACAAGGCCCGGCGCGAGCAGGCCGAGGCCCGCAACAGCGCCGAGCAGC  
TGGCGTACTCGGTGGACAAGATCCTCACCGACAATGACGACAAGCTGCCGGAAGAGGTC  
AAGACGGAGGTCAAGGCCGACGTCGGGGCGCTCAAGACCGCGCTGGCCGGCACCGATGA  
GGACGCGGTTCGAGGCGGCCTCGGAGAAGCTGCAGGCTTCGCAGACCAAACTCGGCGGAG  
CGATTTACGCTTCGGCCCAGGCCGAGGGTGCCGCCGCTGCCGGTGACGCCCCGAGCGAAG  
GTGCCAAGCCCGACGAAGACATCGTCGACGCCGAGATCGTGGACGAAGAAGAACCGAAG  
AACGAGAAGAAGTAGTCATGTCCGACCAGAGCCAATCTGATCAGGGCCGCAACCCCGAA  
AAAGACGAAACCGACGTGGACCCGGCAACGGGTCCCGCCGGGGACGTTCCGGAGGAGCA  
GGATCCTTTGGCGCAAGTCGAAGACATCCTGAACAATGCCGAGGTGCCCGCCGACGAGTC  
GGTGGCCCAGGGCGCCGGGCAGGTGGATGCCGCAGAACTCAAGAACGATCTGCTGCGCT  
TGCAGGCCGAATACGTGAACTACCGCAAACGCGTCGAGCGGGACACCAGCCCGGGCCGT  
CGACCACGCGTGCCCTATAGTAAGGGC

Fig.2 SEQ ID NO: 2. Predicted amino acid sequence of *Arthrobacter* hsp70

MSRAVGIDLGTTNSVSVLEGGEPPVIANAEGRTPSVVAFSKSGEVLVGEIAKRQAVNNI  
DRTIASVKRHMGTDWTVGIDDKKYTAQEISARTLMKLKND AESYLGEKVTD AVITVPAYFND  
AERQATKEAGEIAGLNLVRIVNEPTAAALAYGLDKGKEDELILVFDLGGGTFDVSLLLEV GKDD  
DGFSTIQVRATSGDNRLGGDDWDQRIVDYLLNQLKVKGIDLSKDKIALQRLREASEQAKKEL  
SSATSTNISLQYLSVTPDGPVHLDEQLTRAKFQELTADLLERTKKPFQDVIAEAGIKVSDIDHI  
VLVGGSTRMPAVTELVKQLAGGKEPNKGVNPDEVVAVGAALQAGVLKGERKDVLLIDVTPL  
SLGIETKGGVMTKLIERNIAIPTKRSETFTTADDNQPSVAIQVFQGEREFTRDNKPLGTFELT  
GIAPAPRGVPQVEVTFDIDANGIVHVS AKDKGTGKEQSM TITGGSSLSKEDIERMVADAEAH  
AAEDKARREQAEARN SAEQLAYSVDKILTND DDKLP EEVKTEVKADVGALK TALAGTDEDA  
VEAASEKLQASQTKLGGAIYASQAEGAAAAGDAPSEGAKPDEDIVDAEIVDEE EEPKNEKK

predicted pI= 4.70 / predicted size= 66 kDa



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